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13. ABSTRACT *(Maximum 200 words)*

Immunization requires multiple injections of antigens at certain intervals to achieve the protective immune response. This immunization protocol is not practical in general, especially for Army personnel in the field or in remote areas where access to the health care personnel may be difficult. Oral administration of vaccine is the most desirable route of immunization but often results in poor immune response, mostly because of the gastric degradation and inefficient localization of the vaccine to the gut-associated lymphoid tissue. Our objective in this project is to develop a single-dose oral vaccine system. In the Phase I SBIR, we have demonstrated a several fold efficient uptake of nanoparticles (about 100 nm) compared to larger sized microparticles by the Peyer's patch tissue. In addition, the PORT system capsule technology was established and adapted for the gastric protection and selective delivery of the enclosed nanoparticles to the intestine for uptake by the Peyer's patch tissue. No other comparable system with these advantages exists. In addition, an ELISA has been established for anti-SEB antibody detection in our laboratory. Thus, a strong feasibility is demonstrated in the Phase I SBIR for designing a single-dose oral vaccine using nanoparticle-PORT System technology.

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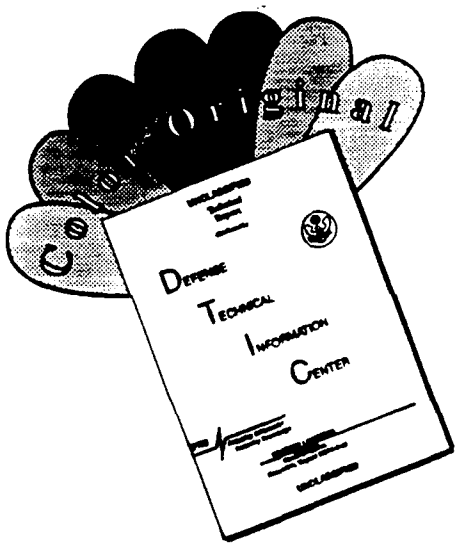

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INTRODUCTION

Current vaccination programs involve, in general, multiple injections at certain intervals to achieve the required protective immune response (1-3). This may not always be practical, especially for Army personnel in the field or those in remote areas where they are prone to certain types of infections and access to health care personnel is often difficult (4, 5). Therefore, an alternative approach is required to protect Army personnel from such infections and toxin exposures that may otherwise result in mortality or morbidity for a considerable period of time. In the Phase I SBIR, we proposed a single-dose oral immunization strategy as an alternative to the current multiple injection immunization programs. Our oral vaccine system consisted of sustained release biodegradable-nanoparticles containing the antigen of interest. Nanoparticles (because of their small size) were hypothesized to more readily and efficiently penetrate the intestinal epithelium and enter the gut-associated lymphoid tissue (GALT), than larger particles. Furthermore, we proposed to deliver the nanoparticle-antigen formulation with our patented programmable oral release technology (PORT System™) capsule which releases the enclosed dosage form in the region of lymphoid tissue of the ileum for optimal uptake by the Peyer's Patches. This time-release capsule-nanoparticle preparation is also hypothesized to enhance immunization efficacy compared to previous preparations by others due to protection of nanoparticles and enclosed antigen from proteolytic enzymes and gastric pH. Staphylococcal enterotoxin B (SEB), is one of a group of enterotoxins produced by S*taphylococcus aureus* which cause food poisoning. SEB-toxoid was used as a model protein-based antigen in the present proposal.

Our working hypothesis is that by virtue of our smaller particle size (nanoparticles Vs. previous microparticles), and the specific delivery and enteric protection offered by the PORT System™, our oral vaccination approach will prove significantly better than those studied previously.

The proposed technology, if successful, will also eventually be applied to other enterotoxins such as streptococcal, salmonella, E. Coli, and other antigens of interest to the Army, either alone or in combinations. Furthermore, oral controlled release nanoparticle systems can induce a secretory immune response (IgA) in addition to a systemic immune response (IgG) (6). This may be particularly useful for the prevention of respiratory, vaginal and gut-associated mucosal infectious diseases. Secretory immune response (IgA) with oral immunization of SEB-toxoid will be helpful in preventing infection through the gut mucossa due to the consumption of contaminated food.

RESULTS OF THE PHASE I WORK

Objectives of the Phase I Effort:

Objectives of the Phase I SBIR application were to test the feasibility of our concept of designing a single-dose oral vaccine system using sustained release antigen loaded nanoparticles enclosed in a PORT System™ time-release capsule. It was hypothesized that nanoparticles will have an efficient uptake by the intestinal lymphoidal tissue, and the PORT System™ capsule will deliver the enclosed nanoparticles at the desired location of the intestine (ileum), thus bypassing degradation of nanoparticles and the enclosed antigen due to gastric proteolytic enzymatic and acid pH. No other systems offer these advantages of protecting and targeting of the antigen in sustained release preparation. The technical objectives in the Phase I SBIR application were:

1) To formulate and characterize a vaccine dosage form consisting of sustained release nanoparticles of polylactic polyglycolic acid co-polymer (PLGA) containing SEB-toxoid. Characterization included *in vitro* studies of SEB-toxoid elution kinetics from PLGA nanoparticles, and acute *in vivo* studies of the *in situ* uptake of formulated nanoparticles by Peyer's patch using a rat intestinal model. **Status**: *Accomplished*

2) To evaluate the PORT System™ time-release "protective" osmotic capsule *in vitro* for the release of enclosed nanoparticles, and *in vivo* in the rabbit gastrointestinal tract and to compare two capsule preparations, which will empty their contained nanoparticles in a burst phase after either 4 or 8 hours. **Status**: *Accomplished*

3) To do immunization feasibility studies in rabbits using two experimental groups:

i) Oral immunization with SEB-toxoid loaded nanoparticles in a PORT System™ time-release capsule,

ii) A single-dose subcutaneous injection of alum-conjugated SEB-toxoid.

Establish ELISA for anti-SEB antibody. **Status**: *ELISA established, immunization study commenced at the conclusion of Phase I. TSRL is committed to sponsor and follow through with this initial immunization study, despite the end of Phase I.*

4) Due to Toxin Technology's extended development time of the SEB-Toxoid, we followed through (at no cost to the DOD) with a tetanus toxoid nanoparticle immunization (subcutaneous single-dose immunization) to demonstrate feasibility of immunization with our nanoparticle technology. **Status:** *Accomplished*

Research Conducted, Findings and Results:

Planning for the Phase II SBIR and and Phase I results were discussed throughout the program period with U.S. Army scientific staff, and in a comprehensive conference call on September 5, 1995 involving Dr. Jack Komisar and the TSRL/University of Michigan group including Dr. John Hilfinger and Dr. John Crison from TSRL, Inc. and Dr. Vinod Labhasetwar and Dr. Robert Levy of The University of Michigan.

Experimental:

1. Formulation of Different Size Bovine Serum Albumin loaded PLGA Microparticles:

All of the initial studies were carried out with formulations containing bovine serum albumin (BSA) as a model protein so that the optimized formulation procedures can be directly used for formulation of SEB-nanoparticles. This was done due to the cost factor of SEB-toxoid. It is hypothesized that nanoparticle formulation parameters will not substantially change by replacing BSA with SEB-toxoid. The emulsification solvent evaporation method was used to formulate different size microparticles to optimize particle size parameter for the uptake by the intestinal tissue in the rat *in situ* intestinal model. All of the formulations also contained 6-Coumarin, a comparatively water insoluble fluorescent dye as a marker for the purpose of quantitation of microparticle following its uptake by the tissue using high pressure liquid chromatography (HPLC) fluorescent detection system.

Multiple emulsion solvent evaporation technique was developed to formulate different sized microparticles. The typical formulation procedure that was developed is as follows: An aqueous bovine serum albumin (BSA, Fraction V, Sigma, St Louis, MO) was emulsified in an organic solution of polylactic polyglycolic acid (PLGA) co-polymer and 6-Coumarin to form a water-in-oil (w/o), a primary emulsion using sonication (Misonix Inc. Model XL 2020™, Farmingdale, NY). The primary emulsion was further emulsified into an aqueous polyvinyl alcohol (PVA) solution to form a multiple water-in-oil-in-water (w/o/w) emulsion. Microparticles of different diameters were obtained by varying concentration and volume of PLGA and PVA solutions, as well as the type of instrument used in the multiple emulsification step (Table I) as a source of energy. Organic solvent was evaporated at room temperature and microparticles were recovered by centrifugation and washed three times with water to remove free BSA and PVA. The formulated particles were lyophilized for 48 hours and stored desiccated at 4°C.

2. Characterization of Microparticles:

i) BSA Loading of Microparticles

In a typical procedure a sample of microparticles (5-10 mg) from each batch was dissolved in 5 ml chloroform. The chloroform layer was then extracted three times with each extraction of 5 ml water. The combined aqueous extracts were assayed for their BSA content with micro BioRad® protein assay.

ii) Particle Size Distribution

Nanoparticles (100 nm) loaded with BSA were characterized for their particle size distribution using a dynamic laser defractometer (NICOMP, Model 370). For other sized particles (500 nm, 1μ and 10 μ), optical microscopy and scanning electron microscopy (SEM) were used to measure an average diameter of the particles.

iii) In Vitro Release of Microparticles

The kinetics of the protein (BSA) release from microparticles was studied under physiologic conditions at 37°C. A 5-10 mg sample of microparticles suspended in 5 ml of phosphate buffer (pH 7.4) was placed in the donor chamber of the double diffusion cells. The cells were separated by 0.1 μm Millipore® membrane (Type: SV). The receiver chamber contained only buffer. The cells were placed in 37°C room on a rotary shaker. The buffer in the receiver side was periodically replaced

with fresh buffer. All the samples were assayed for the released BSA using micro BioRad® protein assay. The experiment was done in triplicate.

3. Peyer's Patches and Nonpatch Tissue Uptake of Microparticles in Rat *in situ* Intestinal Model:

The objective of these acute *in situ* rat experiments was to study the effect of particle size parameter and to assess the optimal particle size for uptake by the Peyer's patch and non patch intestinal tissue. Overnight fasted male Sprague Dawley rats (200-250 g) were anaesthetized with an intraperitoneal injection of sodium pentobarbital (6.5 mg/100 g body weight). The abdomen was opened by a midline incision of 5-6 cm and small incisions were made 15-20 cm apart in the duodenum and ileum. The segments were infused each with 30-40 ml of normal saline to wash off the contents and were ligated to form two loops. An aliquot of the microparticle suspension (4 mg/ml) was infused into these segments using a syringe. After two hours, the loops were opened, microparticle suspension was drained out and the segments were flushed with 40 ml of normal saline. Peyer's patches and non-patch tissues were isolated from the intestine (punched out, 3 mm diameter punch), washed with saline to remove any particles that might have been adhered to the tissue, weighed, and taken for the HPLC analysis of 6-Coumarin as described below. Few tissue samples were used for histological studies for the purpose of localization of microparticles. Length, outer diameter and thickness of the intestinal segment, used for the infusion of the microparticle suspension were also measured to calculate the amount of the total dose of microparticles that each segment of the infused intestine received.

4. Extraction and Analysis of 6-Coumarin from Tissue

Each tissue sample was homogenized separately with 1 ml water using a high speed homogenizer (Stir-Pak®, Lab mixer, Cole-Parmer Instruments Co., Chicago, IL) and each homogenate was then extracted with ethyl acetate five times with each extract of 3 ml. The organic extracts were combined and ethyl acetate was evaporated using vacuum evaporator (SpeedVac® Plus SC110A, Savant Instruments, Inc., Farmingdale, NY). The dry residue was reconstituted in 200 µl acetonitrile and an aliquot was pipetted for HPLC analysis using a 300 mm x 3.9 mm µBondapak C_{18} column with 10 µ packing (Waters, Milford, MA) with scanning fluorescence detector (Waters, Model 470, Milford, MA). A standard curve of 6-Coumarin was constructed for each batch of microparticles .

5. Histological Analysis of Microparticle Localization in Peyer's patch and Nonpatch Tissue:

Histological methodology for the analysis of Peyer's patch and nonpatch tissue to localize microparticle following exposure to microparticles in rat *in situ* experiment has been established and will be carried out during the Phase II SBIR in collaboration with CBL core facility at the University of Michigan.

6 Formulation of SEB-Toxoid Loaded PLGA Nanoparticles

For a single batch of microparticles, 25 mg of SEB-Toxoid (Toxin Technology, Fla) was dissolved in 1.25 ml deionized water. Concurrently, 380 mg PLGA copolymer was dissolved in 12.5 ml methylene chloride. The aqueous SEB-toxoid solution was then emulsified in the PLGA solution to form a water-in-oil (w/o) primary emulsion. Emulsification was acheived using sonication at 65 watts of energy output for 10 minutes using a microtip probe sonicator over an ice bath. This primary emulsion was further emulsified into an aqueous polyvinyl

alcohol (PVA) solution to form a multiple water-oil-water (w/o/w) emulsion. The multiple emulsion was stirred over a magnetic stir plate overnight at 4° C to evaporate the methylene chloride. Microparticles were then recovered by ultra centrifugation (Beckman model XL-70, Arlington Hts., Illinois) at 100,000g and washed three times with water to remove free SEB-toxoid and PVA. The particles were then resuspended in water, lyophilized for 48 hours and stored desiccated at 4°C.

7. Data Analysis:

i) *Calculation of BSA Loading and Encapsulation Efficiency*

The theoretical core loading for a batch of microparticles was based upon the weight of PLGA copolymer and BSA input. Encapsulation efficiency of the formulation was calculated from the actual protein loading and theoretical protein loading.

ii) *Calculation of Microparticle uptake by Tissue*

The amount (μg) of microparticles taken up by an individual Peyer's patches or nonpatch tissue was calculated from the HPLC analysis of 6-Coumarin and the respective standard curves for each batch of microparticles. Number of microparticles taken up by tissue was calculated from their weights in the tissue using the following equation (7):

$$\text{Number of particles} = \frac{w * s * 10^9}{d3}$$

where, w = amount of microparticles in μg taken by tissue

d = diameter of particles in μm

s= specific density of PLGA (1.426)

iii) *Calculation of Efficiency of uptake of Microparticles by the Peyer's Patch and Nonpatch Tissue*

The data of microparticle uptake could be analyzed in three different ways: (i) weight of microparticles taken up per square area of the tissue, (ii) weight of microparticles per unit weight of tissue, and (iii) number of microparticles per unit area or weight of tissue. The most common way of reporting such data in the literature the number of microparticles per unit area of the tissue (8). However, most of these studies were done with polystyrene microparticles where counting the number in tissue cross-sections is the most convenient method of quantitation of uptake of microparticles.

In our studies, efficiency of uptake of microparticles by the Peyer's patch and nonpatch tissue was calculated from the amount of microparticles infused into the intestinal segment and the total surface area of the intestine exposed to the particles for uptake. The total surface area of the intestine exposed to the microparticles was calculated from the length and internal diameter of the intestinal segment into which particles were infused. The total amount of the microparticles infused into this segment was calculated from the volume and concentration of microparticle suspension (4 mg/ml) infused into the segment. Thus, from the theoretical dose calculated as above (6.25 $\mu g/mm^2$) and actual values obtained from the HPLC quantitation, efficiency of uptake of microparticles by the tissue was calculated for each size of microparticles.

iv) Calculation of SEB-Toxoid Loading

SEB-toxoid was analyzed by BioRad Colorimetric procedure. In a typical procedure a sample of 5-10 mg of nanoparticles was dissolved at room temperature in 5 ml chloroform. The chloroform layer was then extracted with three 5 ml volumes of water. The combined aqueous extracts were assayed for their SEB-toxoid content with the micro BioRad protein assay.

v) In vitro Release of SEB-toxoid from Nanoparticles

The kinetics of the toxoid protein release from nanoparticles was studied under physiologic conditions at 37°C. A 5 mg sample of nanoparticles suspended in 5 ml of phosphate buffer (pH 7.4) containing 0.02% sodium azide as an antibacterial agent was placed in the donor chamber of the diffusion cells. The cells were separated by 0.1 um (Type VV) Millipore membrane. 5.0 ml phosphate buffer in the receiver chamber of the diffusion cells was replaced periodically with fresh buffer. All the samples were assayed for released SEB-toxoid using the micro BioRad protein assay as described under SEB-toxoid loading.

8. Development and *In Vitro* Characterization of PORT System™ Capsule:

The PORT System™ is a drug delivery system developed to release a dose of drug in the gastrointestinal tract at a specified time (Figure 1). The design of the PORT System™ is based on controlling the flux of water into a gelatin capsule via a polymer film coating (US patent No 5387421). As fluid enters the capsule, an osmotic charge contained in the base creates a positive pressure that forces the contents of the capsule out. The time of expulsion of the capsule contents is regulated by controlling the flux of water in the capsule, i.e., by varying the thickness of the polymer film coated on the gelatin capsule wall.

As the amount of coating applied to the capsule increases, the permeability and consequently the water flux decreases. The decrease in water flux decreases the rate of pressure build-up within the capsule thereby increasing the pulse time. Our goal in this portion of the project was to formulate and optimize the PORT System for *in vitro* release of enclosed nanoparticles.

i) *Formulation of the PORT System™ Capsule*

Several formulations of the PORT System™ were prepared and tested *in vitro* to optimize the release time and reduce variability. The major variables affecting the release time are the thickness and the density of the cellulose acetate film on the outer wall of the capsule. The polymer coating was administered by dipping capsules in a solution of cellulose acetate in acetone followed by forced air drying. The thickness of the polymer coating was proportional to the number of applied coats and the density was directly proportional to the density of the cellulose acetate/acetone solution.

The osmotic charge in the base of the capsule typically consisted of sorbitol, lactose, Avicel®, magnesium stearate and Explotab®. The concentrations of these constituents were altered for optimized release from the PORT System™ capsule. The test drugs, aspirin (ASA) or acetaminophen (APAP), used in the *in vitro* studies were uniformly dispersed throughout the osmotic charge in the base of the capsule.

PORT System™ - Basic Design

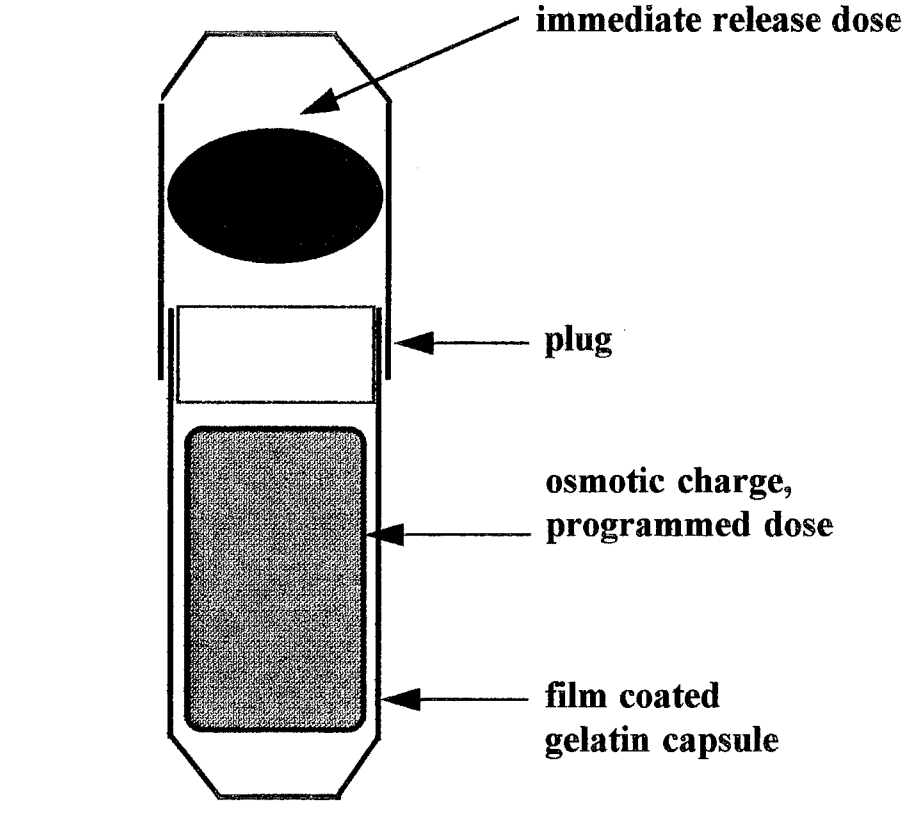


Figure 1. *A Schematic Diagram of the programmed oral release technology or PORT System™.*

Two nanoparticle formulations were tested *in vitro*, one that contained the nanoparticles in a single layer within the base of the capsule and one that contained the nanoparticles evenly mixed throughout the osmotic charge (Figure 2).

For enteric coating of the capsule, 4 coats of either cellulose acetate phthalate, which dissolves at a pH greater than 7.0, or hydroxymethylpropylcellulose phthalate (HP-55), which dissolves at a pH greater than 5.5, were used. **For certain formulations, up to 20 coats (~35% (w/w) total capsule weight) of either of the enteric coatings were used.**

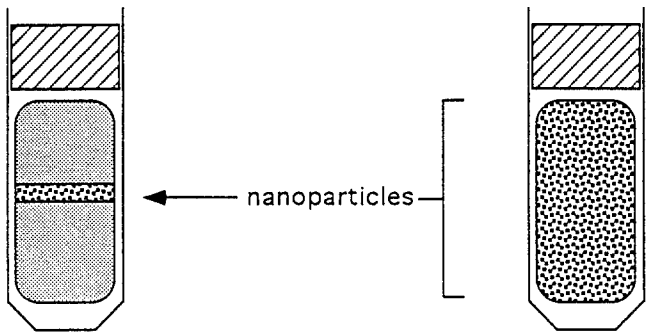


Figure 2. *Schematic of the nanoparticle-PORT System formulation.* Nanoparticles were added to the PORT capsule either as a discreet band or dispersed throughout the osmotic charge.

ii) In vitro pulse time measurement of the PORT System™

To measure the pulse time, capsules were placed in 900 ml of Simulation Intestinal Fluid (SIF), USP, at a pH of 7.5, in a VanKel USP paddle dissolution apparatus at 37°C with a paddle speed of 100 rpm. One ml aliquots were removed at the indicated times, and the samples were assayed for the test drug included in the capsule using established HPLC procedure. The enteric coated capsules were placed in 500 ml of Simulated Gastric Fluid (SGF), USP, at a pH of 1.2, for two hours prior to dissolution in Simulation Intestinal Fluid (SIF). A minimum of 5 capsules were tested for each formulation, the results being reported as the average pulse time± the standard error of the mean.

9. Gastric Transit Time of PORT System™ Capsule in Rabbit:

The objective of this study was to determine gastric transit time of the PORT System™ time-release capsule in rabbit in order to ensure that the capsule formulation is intact in the stomach and releases its content in a pulse as it passes through the intestine.

i) *PORT System™ Formulation:*

This study was performed with a barium sulphate filled PORT System™ capsule. Barium, a radio opaque substance was added to the capsule base as a powder of the PORT System™ capsule (see Figure 1). The gastric emptying time and the pulse time (time at which capsule releases its content) was monitored directly by Fluoroscopy.

ii) *Animal Protocol*

Minor changes in the animal protocols were made in the subsequent protocols tested. These changes in the protocol were based on the observations made during each of the previous protocol tested regarding the PORT System™ gastric emptying and pulse time. In general, overnight fasted New-Zealand Male Rabbits (1.5 kg, SPF) were fed orally single PORT System ™ capsule and periodic Fluoroscopic observations were made to identify the location and status of the capsule in terms of its pulsing.

10. Enzyme Linked Immunosorbent Assay for SEB Antibody (ELISA):

Serum antibody titer was measured by direct ELISA procedure. In brief, the procedure established in our laboratory is as follows: Microtiter plates (Costar Corp., Cambridge, MA) coated overnight with 100 µl (0.5 µg) SEB-Toxin (Toxin Technology Inc., FL) in carbonate buffer (0.05 M, pH 9.6) at 4°C were washed with 0.05 M PBS-Tween (0.1%) (PBS-T) and then blocked with 150 µl of PBS-BSA (2%). The assay was tested with a high titer anti-SEB rabbit serum obtained from Toxin Technology. Serially diluted samples (1:10 to $1:10^8$ in PBS, 100 µl) were added to each well and incubated at 37°C for 60 minutes, washed 3 times with PBS-T and blotted dry. To each well 100 µl of horse radish peroxidase conjugated goat-anti rabbit IgG (1:1000) in PBS-T (0.05%)- BSA (1%) was added and incubated at 37°C for 1 hr. Plates were washed 5 times with PBS-T, blotted dry, and 100 µl of freshly prepared substrate (0.1% 2.2'-Azinobis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium (ABTS) (Sigma) prepared in 0.05 M citrate buffer (pH 4.0) containing 0.001% hydrogen peroxide (Aldrich Chem. Co., Milwaukee, WI) was added. Readings were taken after 30 minutes at 405 nm with back subtraction at 630 nm. All the samples were run in duplicate with appropriate controls, such as coating buffer, conjugate and substrate controls. A similar procedure has been established for quantitation of IgA and IgM antibody levels. Goat anti-rabbit serum is tested for antibodies to SEB before use.

11. ELISA Assay for the detection of SEB-toxin and SEB-toxoid:

SEB-toxin and -toxoid were assayed by a sandwhich ELISA. Microtiter plates (Costar Corporation, Cambridge, MA) were coated with rabbit anti-SEB IgG (Toxin Technology Inc, Fla), diluted 1:1000 in 0.05 M carbonate buffer, pH 9.6. Coating was allowed to occur for at least 18 h at 4°C.

The plates were washed 3 times with phosphate buffered saline with 0.1% Tween-20 washing buffer (PBS-Tween). To each well was added 0.2 ml of PBS-Tween with 3% bovine serum albumin (BSA) and incubated at room temperature for one hour. The microtiter plates were washed three times with PBS-Tween before addition of 0.1 ml test samples or standards containing different concentration of either SEB-toxin or SEB-toxoid in duplicates. The plates were incubated at 37°C for one hour before another washing with PBS-Tween.

The conjugate, horseradish peroxidase-labeled goat-anti rabbit IgG was diluted 1:1000 in PBS-Tween with 1% BSA, and 0.1 ml was added to each well. This was incubated at 37°C for one hour followed by five washings with PBS-Tween. A solution of substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium and 0.003% hydrogen peroxide in 0.05 M citrate buffer, pH 4.0, was added to each well and allowed to react for 10 min. The color intensity was quantitated photometrically at 405 nm by a microplate autoreader (EL 311SX, Bio-Tek® instruments Inc., Winooski VT).

12.

Table I - Immunization Studies in Rabbits

Group	Animals	SEB-Toxoid Dose (ug)	Route-Type of Formulation	Date of Immunization	Samples Collected
I	2	135	SC- SEB Nanoparticles	12/27/95	0,3,7 & 10 weeks
II	5	200	SC- Alum & SEB conjugate	02/08/96	0 and 3 weeks
III	5	200	Oral - Buffered Enteric capsule	02/08/96	0 and 3 weeks
IV	2	200	Oral - PORT capsule	02/20/96	0 week
V	3	0	Oral-Enteric	03/05/96	0 week

i) Description of formulations in each group:

Group I: A suspension of SEB-toxoid loaded nanoparticles in normal saline (20 mg/ml) was prepared by sonication at 45 watts energy for 15 seconds using a microtip probe sonicator. Two rabbits were immunized by subcutaneous injection of 450 ul suspension of SEB-toxoid loaded nanoparticles (Equivalent to 135 ug SEB-toxoid at the loading of 1.5%).

Group II: Alum-SEB toxoid conjugate was prepared by addition of equal volumes of Imject Alum to a solution of SEB-toxoid in sterile water for injection (1 mg/ml) under continuous stirring. The mixture was stirred for 30 minutes after addition of Alum to ensure complete conjugation of SEB-toxoid with Alum. Each rabbit was immunized by subcutaneous injection of 400 ul SEB-toxoid-Alum conjugate (200ul per each SC site).

Group III: All the rabbits in this group were fasted 24 hours prior to the oral immunization. Rabbits were chemically restrained by mild doses of xylazine (5 mg/ml delivered sc.) and ketamine HCL (IMM-25 mg/kg). Enteric coated capsules (#9) filled with SEB-toxoid-loaded nanoparticles mixed with a buffer consisting of dibasic sodium phosphate to maintain a neutral pH were administered orally using a pilling gun. Rabbits were fed normal laboratory diet four hours after dosing.

Group IV: PORT System® time release capsules filled with SEB-toxoid loaded nanoparticles were administered orally as described in Group III.

Group V: Rabbits in this group were orally administered enteric coated capsules filled with control nanoparticles (without SEB-toxoid).

ii) Collection of Serum:

At the interval of 3 weeks, about 2.5-3.0 ml blood was collected from the median artery. Serum was allowed to separate overnight at 4°C. Supernatant serum was centrifuged at 10,000g for 10 min at 4°C and aliquots were stored frozen at -20°C until further analysis.

iii). Collection of Saliva:

About 800 ul saliva was collected from each rabbit at the interval of three weeks following the immunization and was stored frozen at -70°C.

Results:

1. Formulation of Different Sized BSA loaded PLGA Nanoparticles/Microparticles and *In Vitro* Characterization:

As shown in the Table II, different formulation conditions and instrumental energy sources were required to formulate different sized microparticles. However, for making primary emulsion (w/o) sonication was used. This formulation step is one of the steps that determines encapsulation efficiency of the protein. In the multiple emulsification step (water-in-oil-in-water, w/o/w) different energy out put sources were used in addition to the variability in PLGA and PVA concentrations and their volumes to obtain the desired size microparticles.

Table II. Formulation of Different Size Microparticles

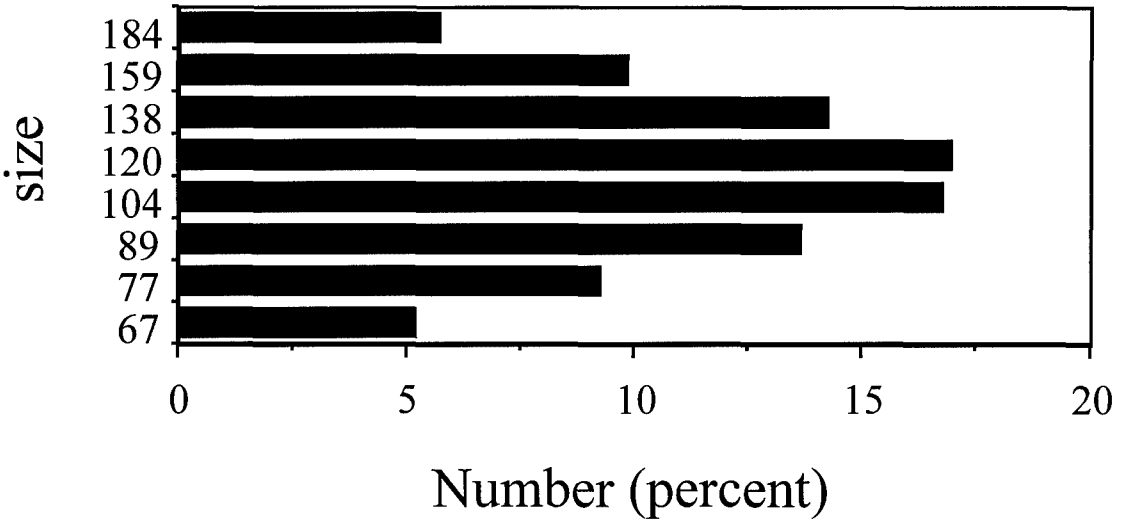
BSA solution (10%)	PLGA solution	PVA solution	Instrument for multiple emulsion	Desired diameter	Actual diameter mean±s.e.m.
0.5 ml	5.0 ml of 3.0%	40 ml of 2.5 %	Sonicator	100 nm	116±4.8 nm
0.5 ml	1.0 ml of 15%	10 ml of 0.5 %	Microfluidizer	500 nm	516±21.9 nm
0.5 ml	2.5 ml of 6%	35 ml of 0.5 %	Homogenizer	1 μm	1.09±.05 μ
0.5 ml	5.0 ml of 3.0 %	30 ml of 2.5 %	Vortex	10 μm	9.44±0.23 μ

BSA loading and encapsulation efficiency of the different size microparticles depended upon the particle size obtained with different formulations (Table III). The maximum BSA loading and encapsulation efficiency was obtained with a 100 nm particle size nanoparticles. Other sized microparticle formulations demonstrated BSA loading of 4.5-6.3% loading. This variation in the BSA loading is expected to be due to the difference in the composition of each type of formulation.

Table III: BSA loading of microparticles (mean±s.e.m., n=3)

Batch size	Actual core loading, wt %	Encapsulation efficiency, %
100 nm	11.7±0.6	47±0.3
500 nm	6.1±0.2	24±0.9
1 μm	4.5±0.5	18±2.1
10 μm	6.3±0.5	24±1.9

Figure 3. Particle Size Distribution of 100 nm Particle



The particle size distribution of 100 nm particle size nanoparticles measured by laser defractometer showed that more than 91% particles were in the range of 70-160 nm with a mean diameter of 116 nm ± 48 (s.d.) (Figure 3). All other size microparticles sizes were measured with optical microscope or scanning electron microscopy. In addition, nanoparticles and microparticles demonstrated particle size distribution within a narrow range and uniform surface characteristics (Figure 4) as evident from the Scanning Electron Microscopy studies.

All sized microparticles showed *in vitro,* an initial burst effect followed by a sustained release over a period of 30 days. With the 100 nm particles the burst release was ~20% of the incorporated protein during an initial 24 hour incubation period and remaining 65% was released by 30 days (Figure 5A). Release patterns of BSA from other sized microparticles was similar but at the slower release rates compared to 100 nm particles (Figure 5C-D).

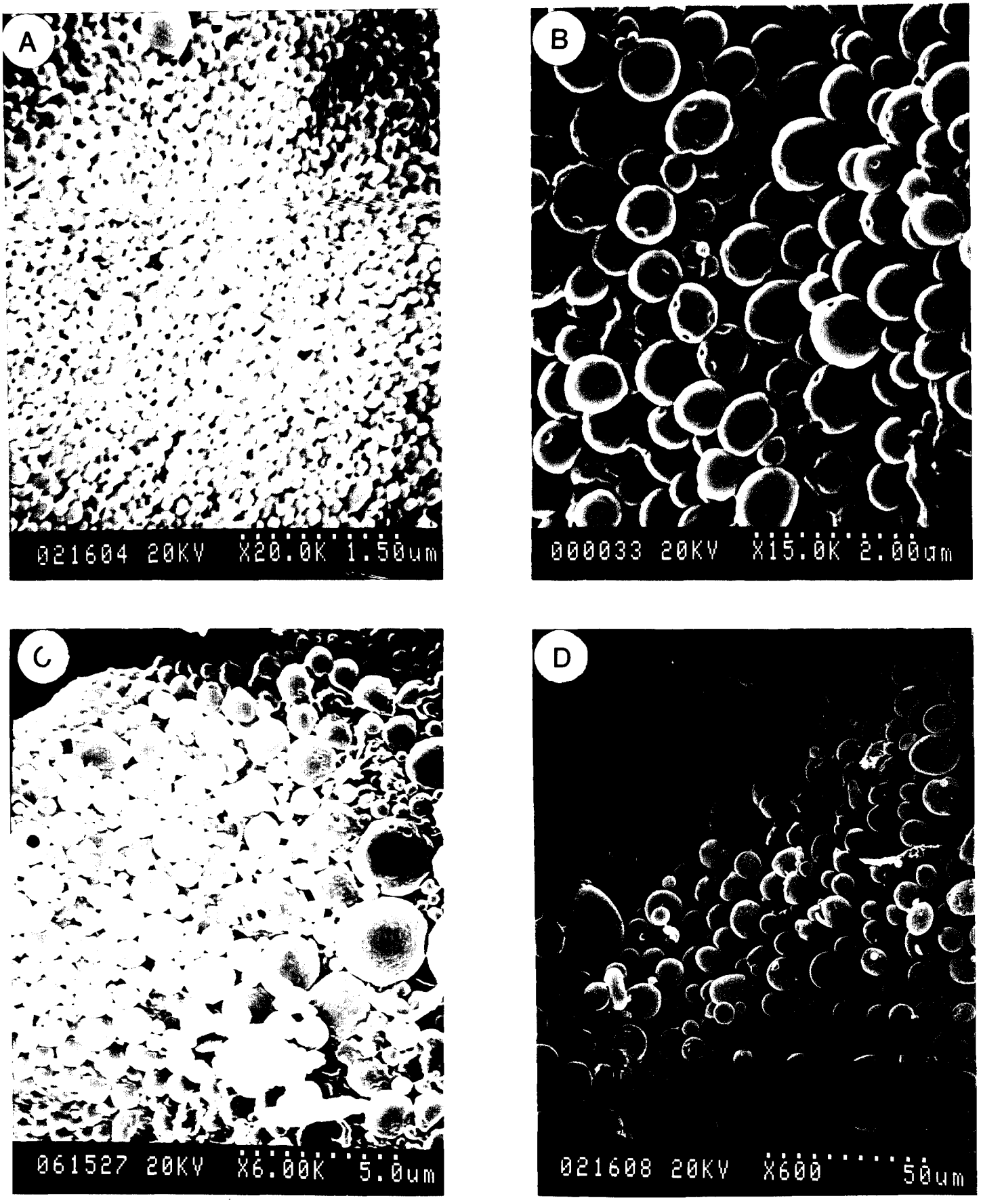


Figure 4. Scanning Electron Micrograph of Different Sized Microparticles.

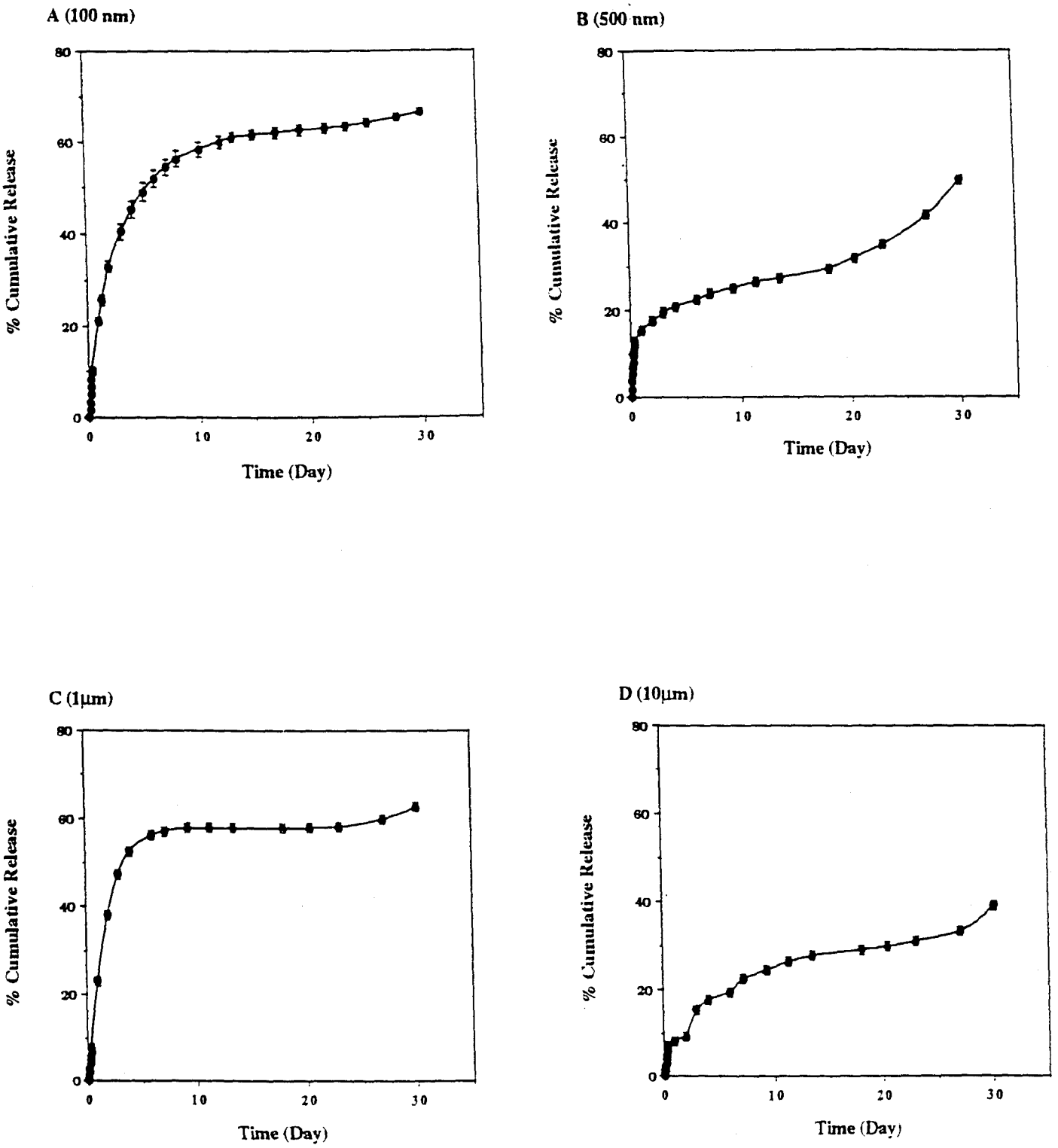


Figure 5. In vitro release of Bovine Serum Albumin from Different Size PLGA Microparticles

2. *In Situ* Uptake of Microparticles In Rat Intestinal Model:

Figure 6 shows the comparative uptake of different sized microparticles in *in situ* rat intestinal model in terms of weight of the microparticle uptake per unit area of the Peyer's patch or nonpatch tissue. It is clearly evident from Figure 6 that the uptake of 100 nm microparticles by the Peyer's patch and nonpatch tissue was several orders of magnitude higher than the uptake of larger sized microparticles. Also evident from the data is that the Peyer's patch tissue from the ileum has selective uptake of microparticles compared to nonpatch tissue. Table IV demonstrates the same data but represented in terms of number of particles per unit area of the tissue.

Table V below demonstrates the efficiency of uptake of different size microparticles by Peyer's patch and nonpatch tissue from duodenum and ileum. The efficiency of uptake was calculated from the theoretical dose (i.e., amount of microparticles infused into the segment) and the actual values obtained for each type of tissue. Irrespective of the method used in computing data, 100 nm microparticles have much higher efficiency of uptake compared to other size microparticles.

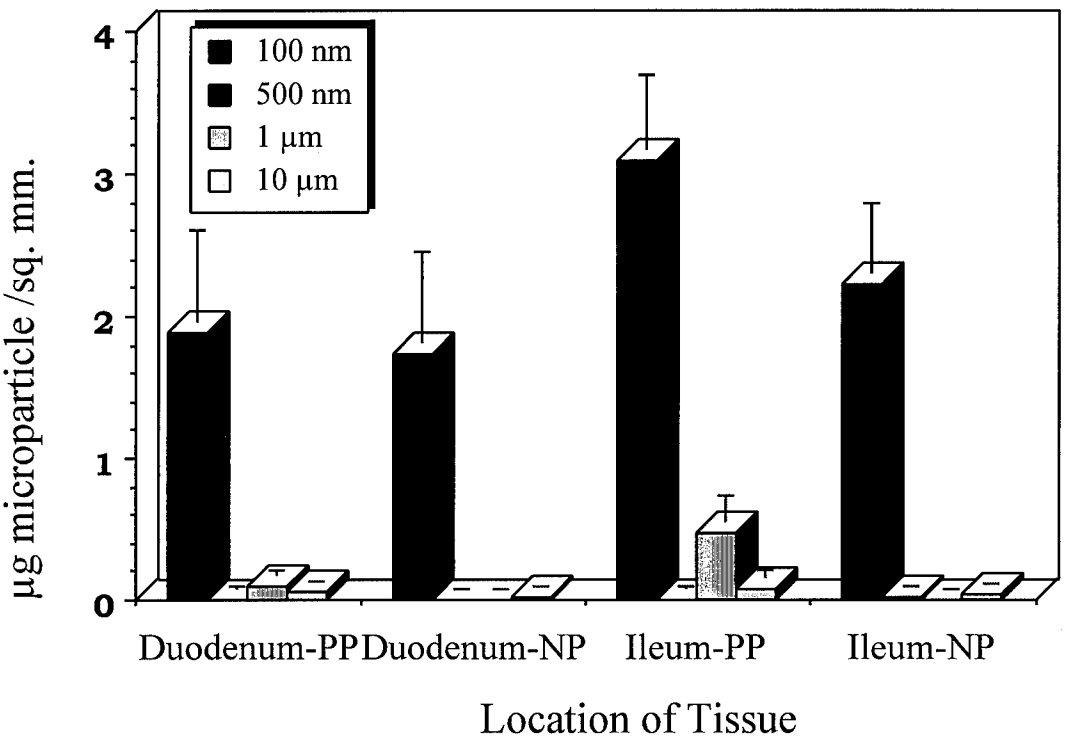
Table IV. Microparticle Uptake in Number/mm^2 from Rat Intestinal Tissue
PP = Peyer's Patch; NP = Non Patch Tissue

Particle Size	Microparticle (Number/mm^2)			
	Duodenum-PP	Ileum-PP	Duodenum-NP	Ileum-NP
100 nm	2.68×10^9	4.41×10^9	2.48×10^9	3.18×10^9
500 nm	8.39×10^4	7.94×10^4	4.39×10^4	1.91×10^5
1μm	1.32×10^5	6.51×10^5	5.90×10^2	1.26×10^3
10 μm	0.70×10^2	1.20×10^2	0.20×10^2	0.40×10^2

Table V. % Efficiency of Microparticle Uptake (Dose = 6.25 μg/ mm^2)

Particle Size	% Efficiency, mean±s.e.m.			
	Duo-PP	Duo-NP	Ileum-PP	Ileum-NP
100 nm	30.07±10.2	27.88±10.3	49.57±8.3	35.69±7.8
500 nm	0.11±0.06	0.06±0.02	0.11±0.06	0.27±0.12
1μm	1.51±0.51	0.007±0.007	7.45±3.05	0.014±0.008
10 μm	0.80±0.19	0.256 ±0.072	1.33±0.69	0.48±0.11

Figure 6. In Situ Uptake of Microparticles in Rat



3) <u>SEB-toxoid Loading of nanoparticles:</u>

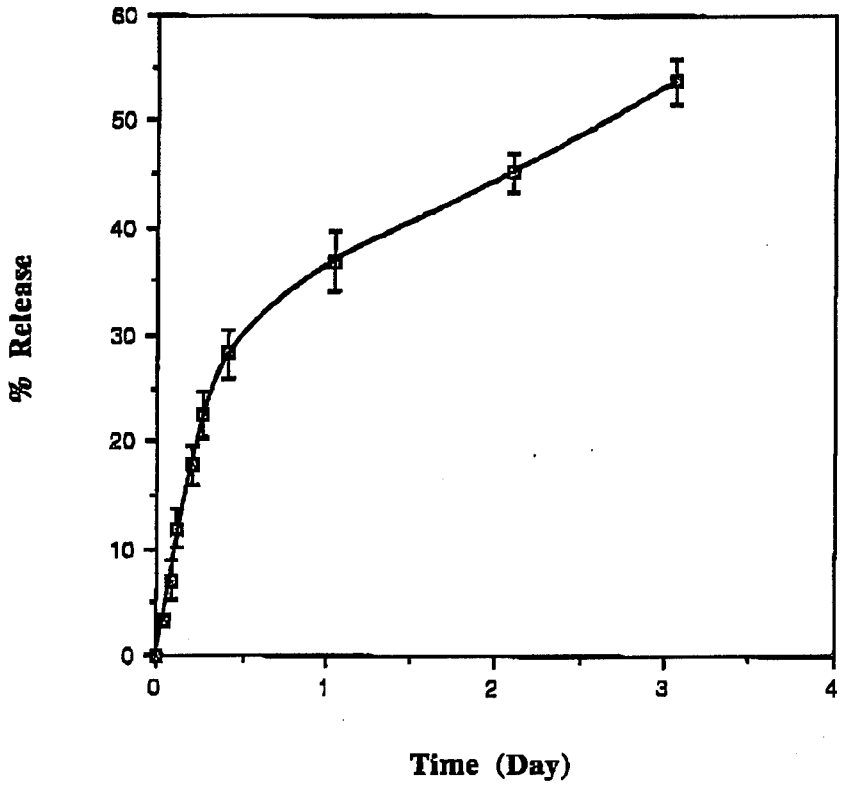
For the determination of SEB-toxoid loading, a standard curve consisting of known concentrations of SEB-toxoid, ranging from 2 ug/ml to 230 ug/ml was run along with the samples. Nanoparticles were analyzed for SEB-toxoid content using the micro BioRad assay. Wt % of theoretical and actual core loading and encapsulation efficiency based on theoretical loading are shown in Table VI

Table VI: SEB-toxoid loading of nanoparticles

Batch size	Actual core loading, wt%	Encapsulation efficiency, %
100 nm	1.54	25%

4. <u>In vitro Release of SEB-toxoid from Nanoparticles</u>

In vitro release studies up to 3 days demonstrated that approximately 30% of the protein was released in an initial burst effect. A total of more than 50% of the protein was released in 3



days (Figure 7). We are continuing these studies over the course of 60 days.

Figure 7 In vitro release of SEB-toxoid from nanoparticles.

5. Formulation and *In Vitro* Characterization of PORT System :

Several formulations of the PORT Systems™ were prepared and tested *in vitro* to optimize the release time and reduce variability. Figures 8A-D show the release times for several formulations tested using acetaminophen (APAP) or aspirin (ASA) as the test drug. Each formulation was tested in Simulation Intestinal Fluid, USP at pH 7.5 using a VanKel USP paddle dissolution apparatus at 37°C with a paddle speed of 100 rpm.

In order to optimize the pulse time of the PORT System™ capsules, the relationship between the number of layers of the cellulose acetate polymer film coating and the release time of the test drug was examined. As seen in Figure 8A, the number of polymer coats applied to the capsule is proportional to the measured release time. These results also clearly depict the pulsing nature of the PORT System™. Figures 8B-C illustrate the reduction in variability in the pulse times achieved through adjusting the concentration of cellulose acetate in acetone applied to the capsule. The average pulse time ± std. dev. in Figure 8B, in which the cellulose acetate concentration was 8% in acetone, is 2.13±0.24 hours. In comparison, the pulse time of the capsule using 9% cellulose acetate in acetone was 2.0 ±0.14 (Figure 8C). This translates to an improvement in the standard deviation from 14.4 minutes to 8.6 minutes.

A problem we encountered using certain formulations of the PORT System™ capsule containing increased osmotic charge was that the test drug was not readily released from the PORT capsule base upon pulsing. However, by proprietary modification of the excipients in the base of capsule, we were able to obtain complete release of the drug after expulsion of the capsule plug (Figure 8D).

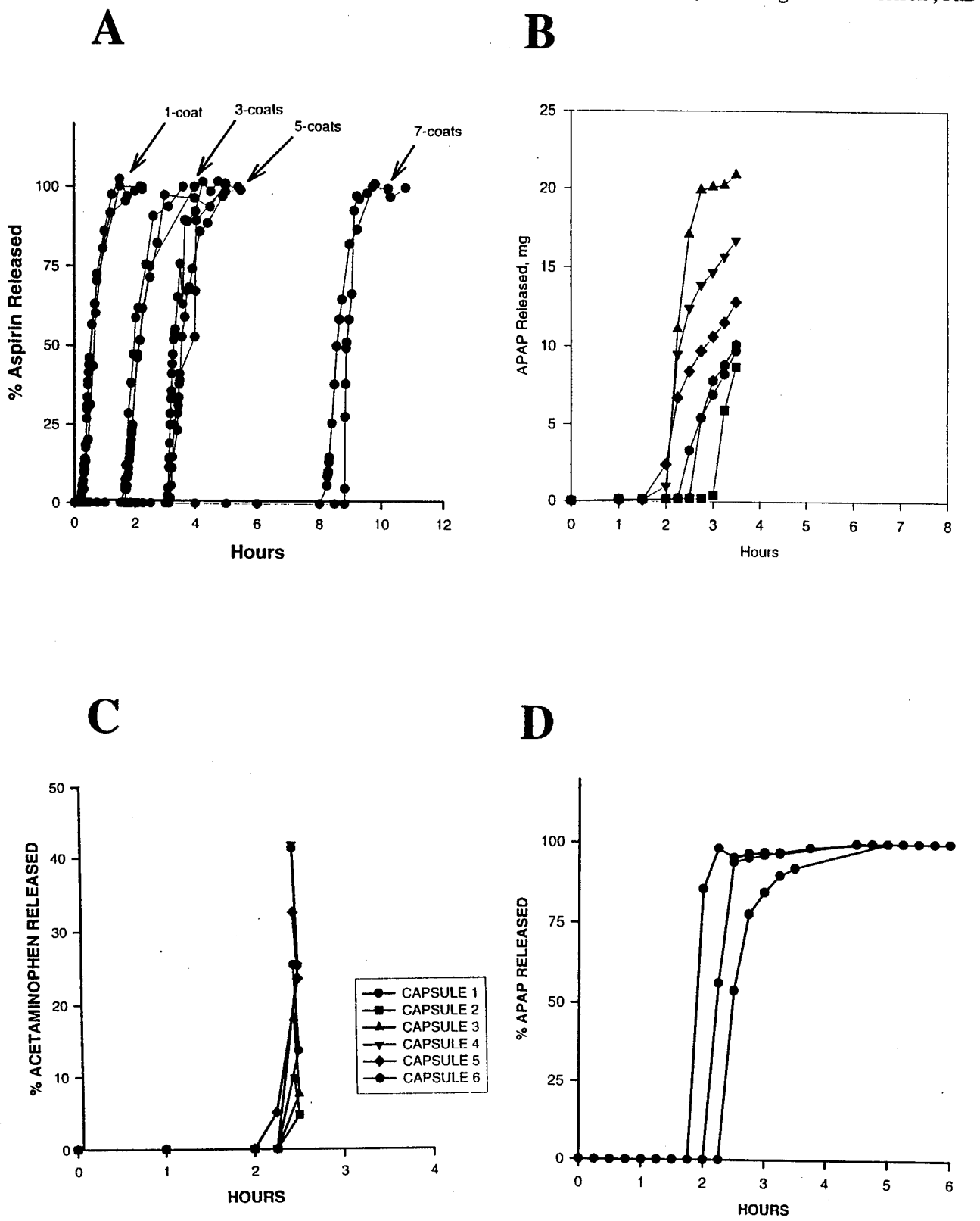


Figure 8. Dissolution profiles for different formulations of the PORT System™. A) Increasing the number of coats of cellulose acetate increases the release time. B) Dissolution profile of 6 capsules coated with 8% cellulose acetate. C) Using 9 % cellulose acetate results in less variability in release times of individual capsules, however not all of the drug is expelled upon pulsing. D) Modification of the excipients in the base of the capsule to increase the 'osmotic energy' results in the release of the entire dose after pulsing of the capsule.

Further formulation optimization was achieved by the addition of a swellable polymer to the osmotic charge/drug dose located in the base of the capsule. As seen in Table VII, the resulting pulse times showed very low variability with an average pulse time of 144.2± 4.4 min.

Table VII: Pulse times of PORT System™ Capsules containing swellable polymer in the osmotic charge formulation.

Formulation	Individual Pulse Time (min)	Ave. pulse Times±sem (min)
100 mg APAP-2 coats Cellulose Acetate PORT™ (SIF, pH 7.5 at 37°C)	142	144.2 ±4.4
	139	
	141	
	160	
	142	
	141	

The enteric coating of the capsule contained 4 coats of cellulose acetate phthalate. The data in Table VIII indicate that the enteric coating remained intact over the course of two hours in Simulated Gastric Fluid, pH 1.2, and that the capsules pulsed as expected after they were moved to Simulated Intestinal Fluid, pH 7.5)

Table VIII. Enteric Formulation of the PORT System

Formulation	Individual Pulse Time (min)	Ave. pulse Times±sem (min)
100 mg APAP - 2 coats Cellulose Acetate, 4 coats Cellulose Acetate Phathalate - PORT System™. (SGF, pH 1.2 at 37oC 2 hrs, SIF pH 7.5.at 37°C)	70	80.2±3.2
	71	
	79	
	80	
	81	
	100	

In the formulation for PORT Systems™ delivery of nanoparticles, we used sorbitol, lactose, or a combination of both in the osmotic charge. The optimum formulation for nanosphere delivery consisted of 50 mg of sorbitol, lactose, Avicel®, magnesium stearate and Explotab® and had a pulse time of 72.7±2.5 min. Pulse times for a number of tested formulations are given in Table IX. Two formulations of nanoparticles were tested *in vitro* using 50 mg of lot#1081-91. Initially, we designed the capsule to contain a thin band of nanoparticles embedded within the osmotic charge (see Figure 2). We found that the capsule pulsed improperly and that the nanoparticles would remain as a discreet compact band during the dissolution experiment. However, when the

nanoparticles were dispersed uniformly throughout the osmotic charge, the pulse capsule performed properly, dispelling the nanoparticles at the appropriate time.

Table IX. Pulse times of optimized nanoparticles formulations in the PORT System.

Formulation	ave. pulse time ± s.e.m. (min)	coefficient of variation
lot # 1081-912, n = 6 (50 mg)	72.7±2.5	8.5
lot # 1081-912, n = 6 (60 mg)	44.5±8.5	47.0
lot # 1081-79m n = 6	62.5±9.3	36.6
sorbitol/lactose, 2:1, n=6	84.0±12.6	33.6
sorbitol (only), n=6	57.2±13.8	58.9

6. *In Vivo* Evaluation of PORT System™ Capsule in Rabbit:

The regular size (size 4) PORT System capsule showed a delay in gastric emptying. In fact, by including barium sulfate in our PORT capsule as a means of visualizing the capsule *in vivo* by fluoroscopy, it was the determined that the #4 size capsule remained in the stomach of rabbits up to 48 hours. Therefore, a Mini-PORT System capsule (size 9), approximately 1/4th the size of the original capsule was tested for gastric emptying by direct visualizationusing fluoroscopy. **We tried a number of formulations and feeding regimens to influence the gastric emptying times of the rabbits. The feeding regimens included fed and fasted states, inclusion of a given amount of glucose to stimulate gastric emptying and using stomach-emptying control rabbits, in which the stomach contents are flushed out and the rabbits prevented from caprophagic behaviour by plastic neck collars (9). However, as seen in Table X, gastric emptying in the rabbit remained widely variable, ranging from 4 hours (Protocol V in Table X) to 45 hours (Protocol XV) and we were unable to reproducibly influence this time . This was problematic to the functioning of the PORT system, since *in vitro* experiments showed that extended incubation of PORT System capusles™ in SGF even with extensive enteric coatings (> 35% (w/w) of the capsule) failed to prevent the uptake of water into the capsule base (data not shown). This, in turn, resulted in a failure of the PORT System™ osmotic pressure mechanism. In light of this data, we altered our vaccination protocol to include one group (Group 3 in Table I) that did not include PORT technology. Rather, the oral dosage form simply consisted of nanoparticles mixed with a phosphate buffer packed into an enterically coated #9 capsule. Even with the uptake of water, the formulation would remain neutral and the nanoparticles would not be rapidly hydrolyzed by the gastric fluid.**

7. Enzyme Linked Immunosorbent Assay for anti-SEB Antibody (ELISA):

All the steps in the ELISA procedure have been optimized. Figure 9 demonstrates the antibody titer for IgG, IgM and IgA of a test high titer anti-SEB rabbit serum (obtained from Toxin Technology).

8. ELISA Assay for the detection of SEB-toxoid:

The ELISA recordings of standard curves for SEB-toxin and SEB-toxoid are shown in Figure 10 and 11. A detection limit of 5 ng/ml of SEB-toxin and 100 ng/ml of SEB-toxoid was attained by the assay. The properies of the toxin and the toxoid are given in Table XI.

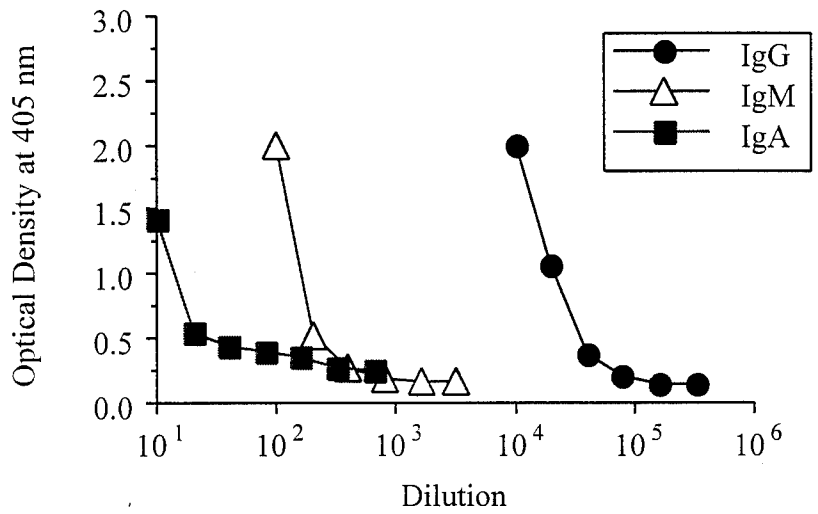
Table X. - Gastric Emptying In the Rabbit

Protocol	PORT System™ Formulation	PORT System™ Size	Rabbit Protocol	Observation
I (8/4/95)	Acetaminophen (APAP) ~95 min. pulse time	#4	Plasma analysis/No food/ 6 hour study	APAP detectable at 3.5 hr in one rabbit and no APAP up to 7 hrs in second.
II (8/8/95)	Acetaminophen ~95 min pulse time	#4	Plasma analysis/No food/10 hour study	APAP not detectable up to 10 hours.
III (8/17/95)	Barium sulfate ~80 min pulse time	#4	X-ray/No food/6 hr study	Intact capsule in two rabbits. Pulsed capsules in 2 hrs in two rabbits (rabbits had chewed the capsules)
IV (8/22/95)	Barium Sulfate ~80 min pulse time	#4	2 g glucose 3 hrs post pilling. Food (2 oz) and 10 ml water at 5 hrs post pilling. 48 hrs study	Capsule intact in stomach for 48 hours.
V (9/6/95)	Barium sulfate	Miniport #9	Food (2 oz) and 10 ml water 4 hours post capsule feeding. 36 hour study.	Gastric emptying 4-5 hours.
VI (9/18/95)	Barium sulfate ~ 72 min pulse time	#9	2 g glucose at 1.5 hrs post pilling. Food (2 oz) and 10 ml water 6 hours post capsule feeding. 24 hour study.	Capsule recovered in feces in one rabbit and no gastric emptying in the other.
VII (10/5/95)	Barium sulfate	#9	Food (2 oz) 2 hours post pilling. 8 hour study.	Capsule pulsed in stomach in one rabbit, remained intact in the second rabbit
VIII (10/12/95)	Barium sulfate ~ 19 min pulse time	#9	Animals fasted for only 4 hours. Food given 2 hours post pilling. 7 hour study	Capsule pulsed in 1.5 hr in one rabbit and 3 hours in intestine in second rabbit.

Table X. continued.

IX (10/23/95)	**Barium sulfate ~50 min pulse time**	**#9**	**4 g glucose at 0.5 hour and food 2 hours post pilling. 30 hour study**	**Capsule pulsed in one rabbit > 10 hrs. Recovered unpulsed in feces in another**
X (11/22/95)	**Barium sulfate ~10 min pulse time**	**#9**	**3 g glucose at 1.0 hour and food at 2 hours post pilling. 30 hour stuydy.**	**Capsule observed intact in stomach in one rabbit and recovered intact in feces in second rabbit.**
XI (12/06/95)	**Barium sulfate ~30 min pulse time**	**#9**	**3 g glucose at 10 min., 6 g glucose at 4.5 hr, 3 g glucose at 5.5 hr and 1 oz food at 6.5 hrs post pilling. 9 hrs study.**	**Capsule intact in stomach after 9 hours.**
XII (12/12/95)	**Barium sulfate ~ 18 min poulse time**	**#9**	**3 g glucose at 12 hours and 2 oz of food at 15 hrs post pilling. 43 hour study.**	**Gastric emptying 18-20 hours and pulse time of > 4 hours.**
XIII (12/26/95)	**Barium sulfate ~ 20 min pulse time**	**#9**	**2 oz food at 18 hrs post pilling. 42 hour study**	**Gastric emptying >22 hours.**
XIV (1/5/96)	**Barium sulfate ~20 min pulse time**	**#9**	**2 oz food 15 hours post capsule feeding /44 hour study**	**One capsule pulsed in stomach after 18 hours. One capsule pulsed in intestine and one recovered in feces.**
XV (1/10/96)	**Barium sulfate ~45 min**	**#9**	**2 0z food at 20 hours post pilling. 45 hour study**	**One capsule pulsed in intestine >28 hours, one recovered from feces and one observed in stomach after 45 hours.**
XVI	**Barium sulfate enteric coated only**	**#9**	**stomach-emptying control rabbits. 24 hour studys**	**One capsule remained in stomach for entire time. One capsule disappeared after >16 hours.**
XVII	**Barium sulfate enteric coated**	**#9**	**stomach-emptying controlled rabitts 24 hour study**	**Both capsules remained in stomach for entire length of study.**

Figure 8. ELISA Assay for Anti-SEB Rabbit IgG, IgM and IgA



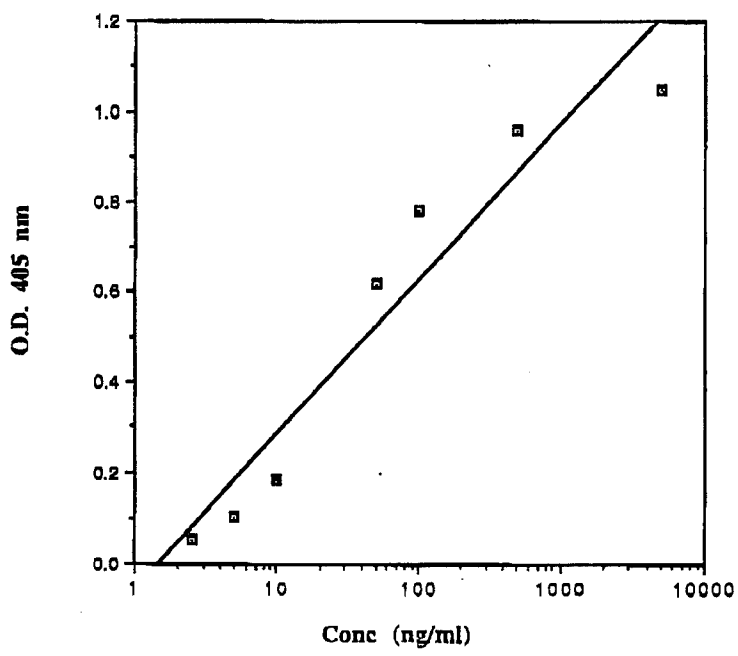


Figure 10. ELISA for SEB-toxin

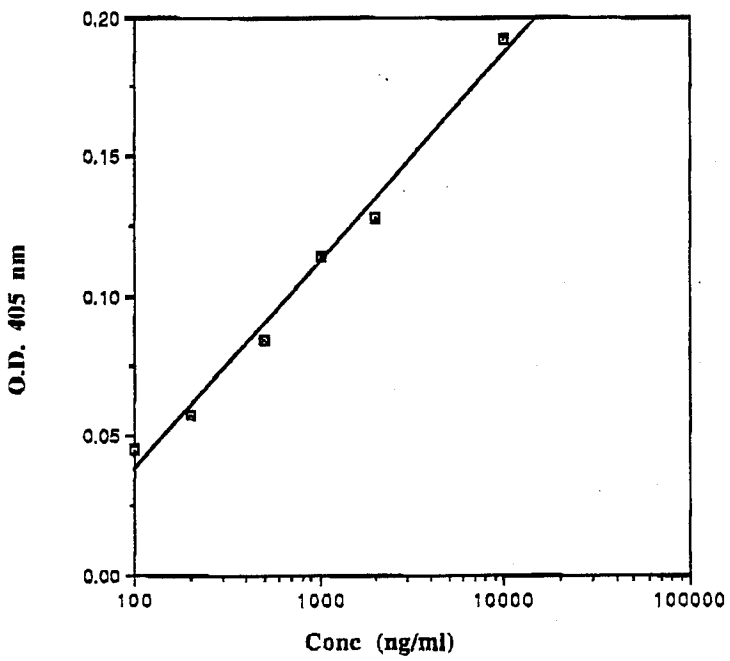


Figure 11. ELISA for SEB-toxoid

Table XI Properties of SEB Toxin and SEB Toxoid

	SEB Toxin	SEB Toxoid
SDS Polyacrylamide gel analysis	> 95 % pure homogeneous band at 28 kD	heterogenous bands - 50 and 75 kD predominant slight bands - 100, 28, <10 kD
Serological[1]		
Ouchterloney	detectable to 5 ug/ml	detectable to ~ 200 ug/ml
ELISA (Ab-toxin-Ab)[2]	detectable to <1 ng/ml	detectable to 10 ng/ml
ELISA (toxin-Ab-Ab)[3]	used as standard	~ 1% of original activity
Mitogenic	to 0.1 ng/ml	to 1ug/ml

[1] All anti-SEB reagents were prepared using the SEB toxin.

[2] Anti-SEB was the capture antibody and anti-SEB conjugated to peroxidase was the detection antibody.

[3] SEB or SEB toxoid was the capture protein, hyperimmune anti-SEB rabbit serum as the primary detection antibody and anti-rabbit IgG conjugated to peroxidase as the secondary detection antibody.

Immunization Studies in Rabbits

Serum samples from Groups I (subcutaneous administration of SEB-nanoparticles) and II (subcutaneous administration of Alum-SEB conjugate) showed high titers of specific IgM and IgG antibodies against SEB-toxoid as early as 3 weeks after immunization (Table XII). In some cases, a slight increase in anti SEB IgA titer was seen (Group II, animal 5). These results clearly show that the encapsulated SEB-toxoid-nanoparticles are fully capable of invoking an immune response against SEB and that the antigenicity of the toxoid is retained after the formulation of the nanoparticles. The average data for Groups I and II are summarized in Figure 12.

Groups III (Enterically coated capsules) showed no increase in IgG anti-SEB levels (data not shown) over a 3 week period and only a very slight rise in the IgM anti-SEB levels. Group IV (PORT System capsules) showed no increases to date (data not shown). Analysis of 3 and 7 week saliva samples showed IgA, IgM and IgG levels similar to the baseline (0 day) values in all the groups (not shown). We feel that it is too early to predict whether or not the oral formulations (Groups III and IV) will invoke a strong immune response. As such, sampling of blood and saliva will continue.

Table XII - Serum analysis for anti-SEB antibody in orally and subcutaneous vaccinated rabbits.

Group	Rabbit #	Time	IgG		IgM		IgA	
		(weeks)	Ab titer	O.D. $_{405}$	Ab titer	O.D. $_{405}$	Ab titer	O.D. $_{405}$
I	1	0	1:10	n.a.	1:10	n.a.	1:10	n.a.
S.C-		3	1:100	>2.0	1:10	0.778	1:10	0.09
nanoparticle		7	1:1000	1.2	1:10	1.092	1:10	0.11
		10	1:50	1.0	1:10	0.995	1:10	0.07
	2	0	1:10	n.a.	1:10	n.a.	1:10	n.a.
		3	1:10	0.983	1:10	0.971	1:10	0.107
		7	1:10	0.449	1:10	>2.0	1:10	0.086
		10	1:10	1.459	1:10	0.737	1:10	0.09
II	1	0	1:10	0.097	1:10	0.804	1:10	0.053
		3	1:1000	>2.0	1:10	1.277	1:10	0.116
SC	2	0	1:10	0.082	1:10	0.245	1:10	0.059
alum-SEB		3	1:1000	1.530	1:10	0.672	1:10	0.092
	3	0	1:10	0.067	1:10	0.257	1:10	0.048
		3	1:500	1.304	1:320	0.974	1:10	0.096
	4	0	1:10	0.092	1:10	0.251	1:10	0.053
		3	1:1000	>2.0	1:10	0.761	1:10	0.124
	5	0	1:10	0.094	1:10	0.272	1:10	0.053
		3	1:1000	>2.0	1:160	1.323	1:10	0.551
III	1	0	1:10	0.065	1:10	0.196	1:10	0.054
		3	1:10	0.037	1:10	0.452	1:10	0.093
Oral	2	0	1:10	0.062	1:10	0.191	1:10	0.060
enteric capsule		3	1:10	0.039	1:10	0.252	1:10	0.088
	3	0	1:10	0.164	1:10	.255	1:10	0.059
		3	1:10	0.062	1:10	0.400	1:10	0.086
	4	0	1:10	0.061	1:10	0.174	1:10	0.050
		3	1:10	0.037	1:10	0.393	1:10	0.087
	5	0	1:10	0.069	1:10	0.322	1:10	0.053
		3	1:10	0.043	1:10	0.862	1:10	0.104

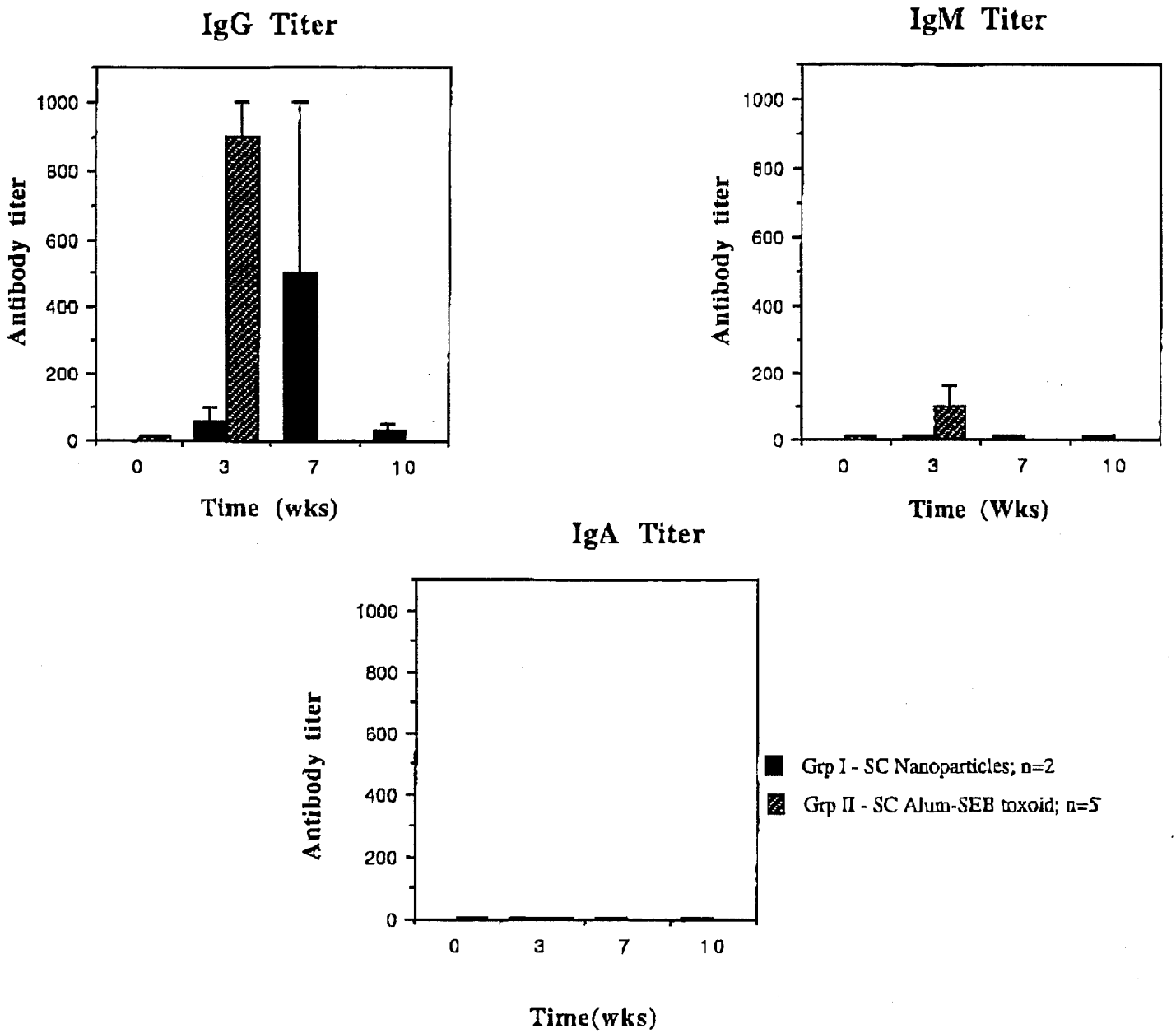


Figure 12. IgG, IgM, IgA antibody titer against SEB-toxoid after subcutaneous immunization of rabbits with either SEB-toxoid nanoparticles or Alum-SEB toxoid conjugate.

Studies in Progress:

1. Immunization Studies:

We will continue to monitor blood and saliva samples on the rabbits. If the orally dosed animals fail to mount any immune response against the SEB-toxin, we will redose with similar formulations.

CONCLUSIONS

1) The study has demonstrated a major important finding that smaller size microparticles (~100 nm) are taken up more efficiently by the intestinal tissue compared to larger size particles investigated in this study. This has significant importance in improving the uptake of the antigen given orally in microparticulate form. In earlier studies by other investigators (10,11), microspheres in the size range of 3-10 μm were used for oral administration of antigen. In general, oral administration resulted in poor uptake of antigen and hence resulted in a weak immune response. Our study clearly demonstrates that smaller size microparticles have a very high efficiency of uptake. Thus, nanoparticle enclosed antigen uptake by the intestinal tissues will also be improved by several fold. This is hypothesized to have a direct effect on levels and duration of the immune response.

2) The SEB-toxoid loading and ELISA assay of serum in different groups of rabbits show that we have been able to successfully encapsulate the SEB-toxoid in nanoparticles by the emulsification solvent evaporation method.

3) Following encapsulation in polymeric particles, the SEB-toxoid induces an immune response against SEB toxoid after subcutaneous administration of nanoparticles, indicating that the antigenicity of the SEB-toxoid is retained after formulation in the nanoparticles.

4) A sandwich ELISA for detection of SEB-toxoid with a detection limit of 100 ng/ml has been developed.

5) It is too early to predict whether the oral formulations of SEB-toxoid- nanoparticles will invoke a strong immune response. Collection of serum and saliva will continue.

6) We must conclude that the rabbit is not an appropriate animal model for testing and developing the PORT system capsule for delivery of nanoparticles to the gut-associated lymphatic tissue. The gastric emptying time is too long and unpredictable. However, we have confidence that in an appropriate animal model, the PORT system capsule would function to selectively deliver the SEB-toxoid nanoparticle to the gut associated lymphoid tissue and we will continue to work in that direction.

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APPENDIX

Contents

Principal Investigator: John Crison , PhD

TOXIN TECHNOLOGY, INC.

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RAOUL F. REISER "Tox Tech" R.H. DEIBEL

John Hilfinger
TSRL
1-313-663-3607

October 10, 1995

Please find the following information regarding the toxoided Staphylococcal enterotoxin B (SEB), lot number 995BX, which was recently sent to you :

The SEB was purified according to the method of Schantz et al. The SEB toxoid was prepared with a glutaraldehyde - lysine method at a pH of 8.0. Please find the following data :

	SEB TOXIN	SEB TOXOID
1) PAGE	> 95 % pure - homogenous band at 28,000 D	heterogenous bands - 50,000 and 75,000 D predominant - slight bands at 100,000, 28,000 and < 10,000 D
2) Serological *		
Ouchterloney	detectable to 5 ug / ml	detectable to approx. 200 ug / ml
EIA (ATA)	detectable to < 1 ng / ml	detectable to 10 ng / ml
EIA (TAA)	used as standard	approx. 1% of original activity
3) Mitogenic	to 0.1 ng / ml	to 1 ug / ml

For the serological assays, all anti-SEB reagents were prepared using the SEB toxin. The ATA - EIA (antibody-toxin-antibody) involved using anti-SEB as the capture antibody and anti-SEB conjugated to peroxidase as the detection antibody. The TAA - EIA (toxin-antibody-antibody) involved using the SEB or toxoided SEB as the capture protein , hyperimmune anti-SEB rabbit serum as the analyte, and anti-rabbit IgG conjugated to peroxidase as the detection antibody.

I am sending the PAGE photographs today.

If there are any questions, please let me know. Thank you.

Sincerely,

Paul F Bina

Paul F. Bina, Toxin Technology

Received 2/8/2000

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 21 Jan 00

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCA, 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for the attached Awards. Request the limited distribution statements for Accession Document Numbers listed be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Virginia Miller at DSN 343-7327 or by email at virginia.miller@det.amedd.army.mil.

FOR THE COMMANDER:

Phylis Rinehart

Encl
as

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management